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(54) Title: BREAD-IMPROVING ADDITIVE COMPRISING A XYLANOLYTIC ENZYME

(57) Abstract

The present invention relates to bread-improving additives. More specifically, the invention relates to bread-improving additives comprising xylanolytic enzymes derived from strains of *Thermomyces lanuginosus*, to methods for preparing baked products, and to methods for improving the baking properties of flour or dough.

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BREAD-IMPROVING ADDITIVE COMPRISING A XYLANOLYTIC ENZYME

TECHNICAL FIELD

The present invention relates to bread-improving additives. More specifically, the invention relates to bread-improving additives comprising xylanolytic enzymes derived from strains of *Thermomyces*, to methods for preparing baked products, and to methods for improving the baking properties of flour or dough.

BACKGROUND ART

In bread-making processes it is known to add bread-improving compositions to the flour or to the bread dough, the action of which, *inter alia*, results in improved texture, volume, flavor and freshness of the bread, as well as improved machinability of the dough.

In recent years, xylanases have gained considerable importance for use in the preparation of bread and baked products, in particular in order to increase the volume and anti-staling of bread and other baked products.

Thus, EP 396,162, EP 493,850 and EP 487,122, relate to bread improvers, deep-frozen dough and fat-free pastry mix, respectively, which comprises xylanolytic enzymes optionally in combination with other enzymes. WO 91/18977 discloses a method of preparing a pentosanase-containing preparation having increased baking activity. EP 321,811 relates to the use of glucose oxidase in combination with a cellulase and/or hemicellulase (such as xylanase) and optionally sulfhydryl oxidase. WO 94/04035 discloses the use of lipase in the preparation of dough and baked products. WO 94/04035 also suggests use of a combination of a lipase and a *Humicola insolens* xylanase, derived from the strain DSM 1800.

Xylanase preparations obtained from strains of the fungus *Thermomyces lanuginosus* (which was formerly classified *Humicola lanuginosa*) have been described [cf. *Lischnig T, Purkarthofer H and Steiner W*; Biotechnology Letters 1993 15 (4) 411-414; *Gomes J, Purkarthofer H, Hayn M, Kapplmüller J, Sinner M, and*

Steiner W, Appl. Microbiol. Biotechnol. 1993 39 700-707]. However, the use of a *Thermomyces lanuginosus* xylanase in bread-improving additives has never been disclosed.

SUMMARY OF THE INVENTION

According to the present invention it has now been found that xylanolytic enzymes derived from strains of *Thermomyces lanuginosus* possess excellent bread-improving properties when added to the flour or to the dough during the manufacture of baked products. Therefore the invention essentially relates to the use of xylanolytic enzymes derived from a strain of *Thermomyces lanuginosus* for improving the properties of a baked product.

Moreover it has been found that the xylanolytic enzymes derived from *Thermomyces lanuginosus* are especially well suited for combinations with amylolytic enzymes with respect to improving the properties of a baked product.

Accordingly, in its first aspect, the present invention provides a bread-improving additive comprising a xylanolytic enzyme derived from a strain of *Thermomyces lanuginosus*.

In another aspect, the invention provides a method of preparing a baked product, which method comprises adding to the flour or to the dough the bread-improving additive of the invention.

In a third aspect, the invention provides a method of improving the baking properties of flour and/or dough, which method comprises adding to the flour and/or to the dough the bread-improving additive of the invention.

DETAILED DISCLOSURE OF THE INVENTION

Bread-Improving Additives

The present invention provides a bread-improving additive comprising a xylanolytic enzyme derived from a strain of *Thermomyces lanuginosus*.

As defined herein, the term "bread-improving additive" comprises dough compositions, dough additives, dough conditioners, pre-mixes, and similar

preparations conventionally used for adding to the flour and/or the dough during processes for making bread or other baked products.

The xylanolytic enzyme may be provided and included in the bread-improving additive in any suitable form, e.g. in the form of a dry powder or a granulate, in particular a non-dusting granulate, a liquid, in particular a stabilized liquid, or a protected enzyme. Granulates may be produced, e.g. as disclosed in US 4,106,991 and US 4,661,452, and may optionally be coated by methods known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding nutritionally acceptable stabilizers such as a sugar, a sugar alcohol or another polyol, lactic acid or another organic acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The xylanolytic enzyme may be added to the flour, to the dough, or to any mixture of flour or dough ingredients. The xylanolytic enzyme may be added in any step of the dough preparation or may, where appropriate, be added in one or multiple steps.

Normally, for inclusion in pre-mixes or in flour, it is advantageous to provide the xylanolytic enzyme in the form of a dry product, e.g. a non-dusting granulate, whereas for inclusion together with a liquid the enzyme is advantageously provided in liquid form.

The bread-improving additive of the invention may comprise any conventional bread-improving agents, and may comprise one or more of the following constituents: A milk powder (providing crust color), an emulsifier (in particular mono- or diglycerides, e.g. DATEM and SSL, diacetyl tartaric acid esters of mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters of monoglycerides, acetic acid or citric acid esters of monoglycerides, polyoxyethylene stearates, phospholipids and lecithin), granulated fat (for dough softening and consistency of bread), and oxidant (added to strengthen the gluten structure; e.g. ascorbic acid, potassium bromate, potassium iodate or ammonium persulfate), an amino acid (e.g. cysteine), a sugar, salt (e.g. sodium chloride, calcium acetate, sodium sulfate or calcium sulfate serving to make the dough firmer) and gluten (to improve the gas retention power of weak flours).

Typically, a bread-improving additive is added in an amount corresponding to of from about 0.1 to 5%, preferably of from about 0.5 to 3% of the added flour.

Bread-improving additives are contemplated useful for e.g. increasing the volume and improving anti-staling properties of a baked product without, however, severely imparting the machinability of the dough. An improved machinability is particularly important for dough types to be processed industrially, e.g. dough types which are to be extruded (e.g. for the preparation of biscuits or other crisp products).

The Xylanolytic Enzyme

The present invention provides a bread-improving additive comprising a xylanolytic enzyme derived from a strain of *Thermomyces lanuginosus*.

In the context of this invention a xylanolytic enzyme is an enzyme having endo-1,4-β-xylanase activity (EC 3.2.1.8).

As defined herein, the term "derived from" encompasses a xylanolytic enzyme obtained from a strain of *Thermomyces lanuginosus*, either produced by and recovered from such strain, or encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. The term also encompasses a xylanolytic enzymes homologous with a *Thermomyces lanuginosus* xylanase, but derived from other microbial sources.

Thermomyces lanuginosus was formerly classified as *Humicola lanuginosa*. Several samples of *Thermomyces lanuginosus* have been deposited and are publicly available from International depository authorities recognized under the Budapest treaty, e.g. American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, USA.

Preferred strains of *Thermomyces lanuginosus* are ATCC 16455, ATCC 22070, ATCC 22083, ATCC 26909, ATCC 26910, ATCC 28083, ATCC 34626, ATCC 36350, ATCC 38905, ATCC 44008, ATCC 46882, ATCC 58158, ATCC 58159, ATCC 58160 and ATCC 66531.

A strain of *Thermomyces lanuginosus* has been deposited according to the Budapest Treaty on the International Recognition of the Deposit of

Microorganisms for the Purposes of Patent Procedure at Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Mascheroder Weg 1b, DE-3300 Braunschweig, Germany, on 4 May 1987, and allotted the Accession No. DSM 4109.

In a preferred embodiment, the xylanolytic enzyme is derived from the strain *Thermomyces lanuginosus* DSM 4109, or a mutant or a variant thereof.

A strain of *Saccharomyces cerevisiae* DSM 10133, containing plasmid DNA comprising the full length DNA sequence presented as SEQ ID NO: 1, encoding a xylanolytic enzyme of the invention, in the yeast vector pYES 2.0, was deposited according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at Deutsche Sammlung von Mikroorganismen und Cellkulturen (DSM), Mascheroder Weg 1b, DE-3300 Braunschweig, Germany, on 19 July 1995, and allotted the Accession No. DSM 10133.

In another preferred embodiment, the xylanolytic enzyme is a xylanolytic enzyme derived from *Thermomyces lanuginosus*, having the amino acid sequence presented as SEQ ID NO: 2, or any partial sequence hereof. The preparation of this enzyme is described in the experimental part of this specification.

In yet another preferred embodiment, the xylanolytic enzyme is a xylanolytic enzyme derived from *Thermomyces lanuginosus*, having the amino acid sequence homologue to the sequence presented as SEQ ID NO: 2. Homology is discussed below.

Methods of Producing the Enzyme

The xylanolytic enzyme may be obtained from the microorganism in question by use of any suitable technique. In particular, the xylanolytic enzyme may be obtained by fermentation of a xylanase producing microorganism in a suitable nutrient medium, followed by isolation of the enzyme by methods known in the art.

The broth or medium used for culturing may be any conventional medium suitable for growing the host cell in question, and may be composed according to the principles of the prior art. The medium preferably contain carbon and nitrogen sources and other inorganic salts. Suitable media, e.g. minimal or complex media, are available from commercial suppliers, or may be prepared

according to published receipts, e.g. the American Type Culture Collection (ATCC) Catalogue of strains.

After cultivation, the enzyme is recovered by conventional method for isolation and purification proteins from a culture broth. Well-known purification procedures include separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, and chromatographic methods such as e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, etc.

Preferably the xylanolytic enzyme is obtained by use of recombinant DNA techniques, known and used by the person skilled in the art. Such method usually comprises cultivation of a host cell, which has been transformed with a recombinant DNA vector capable of expressing and carrying a nucleotide sequence encoding the xylanase in question, in a culture medium under conditions permitting the expression of the enzyme and recovering the enzyme from the culture.

The nucleotide sequence encoding the xylanolytic enzyme according to the invention may be of any origin, e.g. a cDNA sequence, a genomic sequence, a synthetic sequence or any combination thereof. In particular the nucleotide sequence encoding the xylanolytic enzyme according to the invention may be the sequence shown in SEQ ID NO: 1 or an analogue hereof.

As defined herein, a sequence analogue to the sequence presented in SEQ ID NO: 1 may be any nucleotide sequence encoding an enzyme exhibiting xylanolytic activity, which sequence

(i) is homologous to the xylanase encoding part of the DNA sequence presented as SEQ ID NO: 1, or to the DNA sequence obtainable from the plasmid in the strain *Saccharomyces cerevisiae* DSM 10133; or

(ii) hybridizes with the same oligonucleotide probe as the xylanase encoding part of the DNA sequence presented as SEQ ID NO: 1, or with the DNA sequence obtainable from the plasmid in the strain *Saccharomyces cerevisiae* DSM 10133; or

(iii) encodes a polypeptide which is at least 70% homologous to the polypeptide encoded by the DNA sequence presented as SEQ ID NO:

1, or to the DNA sequence obtainable from the plasmid in the strain *Saccharomyces cerevisiae* DSM 10133; or

(iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified xylanase derived from the strain *Thermomyces lanuginosus*, DSM 4109, or encoded by the DNA sequence presented as SEQ ID NO: 1, or the DNA sequence obtainable from the plasmid in the strain *Saccharomyces cerevisiae* DSM 10133.

As defined herein, a DNA sequence analogue to the xylanase encoding part of the DNA sequence presented as SEQ ID NO: 1 is intended to indicate any DNA sequence encoding a xylanolytic enzyme, which enzyme has one or more of the properties cited under (i)-(iv), above.

The analogous DNA sequence may preferably be isolated from another or related (e.g. the same) organism producing the xylanase component, on the basis of the xylanase encoding part of the DNA sequence shown in SEQ ID NO: 1, or a suitable subsequence (such as 20-500 bp) thereof, e.g. using the procedures described herein, and thus, e.g. be an allelic or species variant of the DNA sequence comprising the DNA sequence presented herein.

Alternatively, the analogous sequence may be constructed on the basis of the xylanase encoding part of the DNA sequence presented as SEQ ID NO: 1, or any subsequence thereof, e.g. by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the xylanolytic enzyme encoded by the DNA sequence, but which corresponds to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence.

When carrying out nucleotide substitutions, amino acid changes are preferably of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain. Examples of conservative

substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine). For a general description of nucleotide substitution, see e.g. *Ford et al.*, Protein Expression and Purification, 2 1991 95-107.

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active xylanolytic enzyme. Amino acids essential to the activity of the xylanase encoded by the DNA construct of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (cf. e.g. *Cunningham and Wells*, Science 1989 244 1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological (i.e. proteolytic) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photo affinity labelling (cf. e.g. de *Vos et al.*, Science 1992 255 306-312; *Smith et al.*, J. Mol. Biol. 1992 224 899-904; *Wlodaver et al.*, FEBS Lett. 1992 309 59-64).

It will be understood that the xylanase encoding part of the DNA sequence presented as SEQ ID NO: 1, or any subsequence thereof, may be used as probes for isolating the entire DNA sequence encoding the xylanolytic enzyme, e.g. the DNA sequence presented as SEQ ID NO: 1.

The homology referred to in i) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (*Needleman S B & Wunsch C D*; J. Mol. Biol. 1970 48 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence exhibits a

degree of identity preferably of at least 70%, in particular at least 80%, at least 85%, at least 90%, or even at least 95% to the coding region of the xylanase encoding part of the DNA sequence shown in SEQ ID NO: 1, or to the DNA sequence obtainable from the plasmid in the strain *Saccharomyces cerevisiae* DSM 10133.

The hybridization referred to in (ii) above is intended to indicate that the analogous DNA sequence hybridizes to the same oligonucleotide probe as the DNA sequence encoding the xylanase component under certain specified conditions which are described in more details below. The probe to be used may conveniently be constructed on the basis of the xylanase encoding part of the DNA sequence SEQ ID No. 1, or a sub-sequence thereof encoding at least 6-7 amino acids of the enzyme, or on the basis of the deduced amino acid sequence shown in SEQ ID NO 2. In the latter case the probe is prepared from an amino acid subsequence corresponding to a high number of low degenerated codons.

Normally, the analogous DNA sequence is highly homologous to the DNA sequence such as at least 70% homologous to sequence shown in SEQ ID NO: 1 encoding a xylanase component of the invention, preferably at least 80%, in particular at least 85%, at least 90%, or even at least 95% homologous to the sequence shown in SEQ ID NO: 1.

The degree of homology referred to in (iii) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art, e.g. GAP provided in the GCG program package (*Needleman S B & Wunsch C D*; J. Mol. Biol., 1970 48 443-453). Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the polypeptide encoded by an analogous DNA sequence exhibits a degree of identity preferably of at least 70%, in particular at least 80%, at least 85%, at least 90%, or even at least 95%, to the enzyme encoded by a DNA construct comprising the xylanase encoding part of the DNA sequence shown in SEQ ID NO: 1 or the DNA sequence obtainable from the plasmid in the strain *Saccharomyces cerevisiae* DSM 10133.

According to the method described in the above, the DNA homology of the xylanase of the invention against most prior art xylanases was determined

using the computer program GAP. The xylanase of the invention showed only 63% DNA homology to the xylanase I from *Trichoderma reesei* (*Torronen A et al.*, Biotechnology 1992 10 11 1461-1465), and 63% DNA homology to xylanase I from *Cochliobolus carbonum* (*Apel P C et al*; Mol. Plant Microb. Interact. 1993 6 467-473).

The term "derived from" in connection with property (iv) above is intended not only to indicate a xylanase component produced by the strain *Thermomyces lanuginosus*, DSM 4109, but also a xylanase component encoded by a DNA sequence isolated from this strain and produced in a host cell transformed with said DNA sequence. The immunological reactivity may be determined by the method described below.

Additional Enzyme Activities

While the bread-improving additive may comprise a xylanolytic enzyme as the only enzyme added, the properties of dough and/or baked products may be further improved when the xylanolytic enzyme is used in combination with one or more additional enzymes.

Accordingly, in a preferred embodiment, the invention provides a bread-improving additive comprising a xylanolytic enzyme derived from a strain of *Thermomyces lanuginosus* and one or more additional enzyme(s). The additional enzyme(s) may either be one or more enzymes present in the xylanase preparation recovered from the organism producing it, or may, more preferably, be added to the bread-improving additive.

In a preferred embodiment, the additional enzyme is selected from the group consisting of an amylase, in particular an amyloglucosidase or an α-amylase (at present contemplated useful for providing sugars fermentable by yeast), a peptidase, a maltogenase, a lipase (at present contemplated useful for reducing the dough stickiness caused by xylanase and an over-all volume increase), a cellulase, a hemicellulase, a pentosanase, a glucose oxidase (at present contemplated useful for strengthening the dough), a protease (at present contemplated useful for gluten weakening in particular when using hard wheat flour), and a peroxidase or a laccase (at present contemplated useful for improving dough consistency).

In a more specific embodiment, the additional enzyme is selected from the group consisting of a lipase, an amylase and/or an oxidase.

Moreover it has been found that the xylanolytic enzymes derived from *Thermomyces lanuginosus* are especially well suited for combinations with amylolytic enzymes, in particular amylolytic enzymes of fungal origin, with respect to improving the properties of a baked product. Therefore, in a yet more specific embodiment, the additional enzyme(s) is/are one or more selected from the group consisting of an α-amylase and an amyloglucosidase. Preferably, the α-amylase is derived from a strain of *Aspergillus*, in particular a strain of *Aspergillus oryzae*.

In a further preferred embodiment, the additional enzyme is a lipase.

The additional enzyme is preferably of microbial (bacterial, yeast or fungal) origin and may be obtained by techniques conventionally used in the art.

The amylase may be derived from a bacterium or a fungus, in particular from a strain of *Aspergillus*, preferably a strain of *Aspergillus niger* or *Aspergillus oryzae*, or from a strain of *Bacillus*. Commercially available α-amylases useful for the present purpose are Fungamyl™ (an *Aspergillus oryzae* α-amylase, available from Novo Nordisk A/S, Denmark), Novamyl™ (a *Bacillus stearothermophilus* maltogenic α-amylase, available from Novo Nordisk A/S, Denmark), and BAN™ (a *Bacillus amyloliquefaciens* α-amylase,available from Novo Nordisk A/S, Denmark). The amyloglucosidase may in particular be AMG™ (an *A. niger* amyloglucosidase available from Novo Nordisk A/S, Denmark). Other useful amylase products include Grindamyl™ A 1000 or A 5000 (available from Grindsted Products, Denmark) and Amylase™ H or Amylase™ P (available from Gist-Brocades, The Netherlands).

The glucose oxidase may be a fungal glucose oxidase, in particular as Gluzyme™ (a *Aspergillus niger* glucose oxidase, available from Novo Nordisk A/S, Denmark).

The protease may in particular be Neutrase™ (available from Novo Nordisk A/S, Denmark).

The lipase may be derived from a strain of *Thermomyces*, a strain of *Rhizomucor*, a strain of *Candida*, a strain of *Aspergillus*, a strain of *Rhizopus*, or a strain of *Pseudomonas*. In particular the lipase may be derived from a strain of *Thermomyces lanuginosus*, a strain of *Rhizomucor miehei*, a strain of *Candida*

antarctica, a strain of *Aspergillus niger*, or a strain of *Pseudomonas cepacia*. In specific embodiments, the lipase may be Lipase A or Lipase B derived from a strain of *Candida antarctica* as described in WO 88/02775, or the lipase may be derived from a strain of *Rhizomucor miehei* as described in EP 238,023, or a strain of *Humicola lanuginosa* described in EP 305,216, or a strain of *Pseudomonas cepacia* as described in EP 214,761 and WO 89/01032.

Besides the above mentioned additional enzymes, a microbial produced xylanase preparation may contain varying minor amounts of other enzymatic activities inherently produced by the producer organism in question.

Enzyme Dosage

It is well known when working with pentosanases or xylanases in baking that enzyme activity determination based on soluble or insoluble pentosans or arabinoxylans substrates can not be correlated to the baking performances of different enzyme preparations from different microorganisms [cf. *Joan Qi Si et al.*; Presentation at the AACC Annual Congress, 1993, Miami Beach, Abstract No. 308]. Therefore, the optimum dosage of the xylanolytic enzyme must be determined experimentally.

The optimum dosage may e.g. be defined as the dosage that provides the maximum specific volume increase without producing a too sticky dough. The dosage may be expressed as g enzyme per kg flour, mg protein per kg flour, or a specified enzymatic activity per kg flour.

In the present invention, FXU per kg flour has been chosen as reference activity. However, it must be understood that the dosage of a *Thermomyces lanuginosus* xylanases (when expressed in FXU per kg of flour) cannot be compared directly with the dosage of any other xylanases (although expressed in the same activity), as the activity does not fully correlate with baking performance. Accordingly, when comparing the xylanolytic enzyme of the invention with other xylanases, it is essential that the baking performance (in terms of optimal volume and low dough stickiness) is chosen as the comparative parameter.

It is at present contemplated that a suitable enzyme dosage corresponds to of from about 5 to about 5000 FXU/kg of flour, preferably of from

about 20 to about 2000 FXU/kg of flour. A more preferred enzyme dosage is of from about 50 to about 1500 FXU/kg of flour, particularly of from about 100 to about 1000 FXU/kg of flour.

The additional enzymes may be dosed in accordance with established baking practice. It is at present contemplated that a preferred lipase dosage is in the range of from about 10 to about 100,000 LU/kg of flour, preferably of from about 25 to about 50,000 LU/kg of flour, most preferred of from about 50 to about 5000 LU/kg of flour. Similar it is contemplated that a preferred amylase dosage is in the range of from about 5 to about 500 FAU/kg of flour.

Methods of Preparing Baked Products

In another aspect the invention provides a method of preparing a baked product, which method comprises adding to the flour or to the dough the bread-improving additive of the invention.

As defined herein, the term "baked product" is intended to include any product prepared from dough, in particular a bread product. The baked product may be yeast-leavened or chemically leavened and may be of a soft or a crisp character. Examples of baked products, whether of a white, light or dark type, which may advantageously be produced by the present invention are bread, typically in the form of loaves or rolls, French baguette-type bread, pita bread, tacos, cakes, pan-cakes, waffles, biscuits, crisp bread and the like.

The baked product prepared according to the invention is normally based on wheat meal (including whole meal) or flour, optionally in combination with other types of meal or flour such as corn flour, rye meal, rye flour, oat flour or meal, soy flour, sorghum meal or flour, or potato meal or flour. However, it is contemplated that the method of the present invention will function equally well in the preparation of dough and baked products primarily based on other meals or flours, such as corn meal or flour, rye meal or flour, or any other types such as the types of meal or flour mentioned above. The dough may be substantially free from added fat or may contain even considerable amounts of fat (e.g. butter, margarine, shortening, oil, or the like).

The handling of the flour, the dough, and/or the baking may be carried out by conventional means suitable for preparing the baked product in question. Usually a method of preparing a baked product comprises the steps of kneading of the dough, subjecting the dough to one or more proofing treatments, and baking the product under suitable conditions, i.e. at a suitable temperature and for a sufficient period of time. For instance, the dough may be prepared using a normal straight dough process, a sour dough process, an overnight dough method, a low-temperature and long-time fermentation method, a frozen dough method, the Chorleywood Bread process, or the Sponge and Dough process.

Methods of Improving Baking Properties

The bread-improving additive of the invention may be used a method of improving baking properties of a dough, which method comprises adding to the flour and/or to the dough the bread-improving additive of the invention. Alternatively, the bread-improving additive of the invention may be added to any ingredient of the dough and/or to any mixture of the dough ingredients.

As defined herein, the expression "improved baking properties" is intended to reflect any improvement of the dough quality and/or the quality of the final product. Such improvement may include improved texture, increased volume, improved flavor, improved anti-staling, improved softness, improved crumb softness upon storage, and improved freshness of the baked product, as well as improved machinability of the dough.

The invention also provides a dough or a baked product prepared by use of a bread-improving additive the invention.

Xylanolytic Activity

The endo-xylanase activity may be expressed in "Farbe Xylanase Units" (FXU). A xylanase sample is incubated with a remazol-xylan substrate (4-O-methyl-D-glucurono-D-xylan dyed with Remazol Brilliant Blue R, Fluka) at standard conditions (i.e. 50°C, pH 6.0 and 30 min. reaction time), and the background of non-degraded dyed substrate is precipitated with ethanol. The remaining blue color in the supernatant is determined spectrophotometrically at 585 nm and is proportional to

the endo-xylanase activity. The FXU is determined relatively to an enzyme standard. A folder AF 293.6/1, describing this FXU assay in more details, is available upon request from Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

Lipolytic Activity

Lipolytic activity may be expressed in Lipase Units (LU). One (1) LU is defined as the amount of enzyme which liberates 1 μmol titratable butyric acid per minute under given standard conditions (i.e. tributyrine substrate, 30.0°C, and pH 7.0). A folder AF 95/5, describing this LU-assay in more details, is available upon request from Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

Amylolytic Activity

The α-amylase activity may be expressed in "Fungal α-Amylase Units" (FAU). One (1) FAU is the amount of enzyme which under standard conditions (i.e. at 37°C and pH 4.7) breaks down 5260 mg solid starch (Amylum solubile, Merck) per hour. A folder AF 9.1/3, describing this FAU assay in more details, is available upon request from Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

Hybridization Conditions

Suitable hybridization conditions for determining hybridization between an oligonucleotide probe and an "analogous" DNA sequence of the invention may be defined as described below. A suitable oligonucleotide probe to be used in the hybridization may be prepared on the basis of the xylanase encoding part of the DNA sequence shown in SEQ ID NO: 1, or any sub-sequence thereof, or on the basis of the deduced amino sequence shown in SEQ ID NO: 2. An example of a suitable probe, is the DNA sequence corresponding to the xylanase encoding part of SEQ ID NO: 1, i.e. nucleotides at positions 31-705 in SEQ ID NO: 1.

A filter containing the DNA fragments to hybridize is subjected to presoaking in 5x SSC, and prehybridized for 1 hour at about 50°C in a solution of

5x SSC, 5x Denhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 μg of denatured sonicated calf thymus DNA. After hybridization for 18 hours at ~45°C in the same solution supplemented with 50 μCi 32-P-dCTP labelled probe, the product is washed three times in 2x SSC, 0.2% SDS, for 30 minutes at preferably no more than 55°C, in particular no more than 60°C, no more than 65°C, no more than 70°C, no more than 75°C, preferably no more than 80°C.

Molecules to which under these conditions the oligonucleotide probe hybridizes, may be detected using an x-ray film.

Immunological Cross-reactivity

Antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified xylanolytic enzyme. More specifically, antiserum against the enzyme of the invention may be raised by immunizing rabbits (or other rodents) according to the procedure described by *Axelsen et al.*; A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, (in particular Chapter 23), or by *Johnstone and Thorpe*; Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (in particular pp. 27-31).

Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ($(NH_4)_2 SO_4$), followed by dialysis and ion exchange chromatography, e.g. on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Outcherlony double-diffusion analysis (*Ouchterlony O*; Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706; or *Roitt I*; Essential Immunology, Blackwell Scientific Publications, 1984, pp. 145-147), by crossed immunoelectrophoresis (*Axelsen et al.*, *supra*, Chapters 3 and 4), or by rocket immunoelectrophoresis (*Axelsen et al.*, *supra*, Chapter 2).

EXAMPLES

The invention is further illustrated with reference to the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

A Xylanolytic Enzyme derived from *Thermomyces lanuginosus*
Expression in *Aspergillus*

Donor Organism

mRNA was isolated from *Thermomyces lanuginosus*, DSM 4109, grown in a xylan containing fermentation medium with agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C.

Yeast Strains

The *Saccharomyces cerevisiae* strain used below is JG169 (MATα; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-113; prc1::HIS3; prb1:: LEU2; cir+).

Plasmids

For expression the commercially available yeast plasmid pYES 2.0 (Invitrogen™) was used.

The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775, which was described in EP 238,023. The construction of pHD414 is further described in WO 93/11249.

Extraction of Total RNA

Extraction of total RNA was performed with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion, and isolation of poly(A)$^+$RNA by oligo(dT)-cellulose affinity chromatography using the procedures described in WO 93/11249.

cDNA Synthesis and Modification

Double-stranded cDNA was synthesized from 5 μg of poly(A)$^+$ RNA by the RNase H method (*Gubler U, Hoffman B J*, Gene 1983 25 263-269; *Sambrook et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Lab., Cold Spring

Harbor, NY, 1989) using the hair-pin modification. The procedure is further described in WO 93/11249.

After having been treated with mung bean nuclease, the ds cDNA was made blunt-ended with T4 DNA polymerase (Invitrogen™) and the cDNA was ligated to non-palindromic BstX I adaptors (1 μg/μl, Invitrogen™) in accordance with the manufacturers instructions.

Construction of cDNA Libraries

The adapted, ds cDNA was recovered by centrifugation, washed in 70% EtOH and resuspended in 25 ml H_2O. Prior to large-scale library ligation, four test ligations were carried out in 10 μl of ligation buffer (same as above), each containing 1 μl ds cDNA (reaction tubes #1 - #3), 2 units of T4 ligase (Invitrogen™) and 50 ng (tube #1), 100 ng (tube #2) and 200 ng (tubes #3 and #4) Bst XI cleaved yeast expression vector (either pYES 2.0 vector, Invitrogen™, or yHD13).

Using the optimal conditions, a large-scale ligation was set up in 40 μl of ligation buffer. One μl aliquot were transformed into electrocompetent *E. coli* 1061 cells, and the transformed cells were titered and the library plated on LB + ampicillin plates with 5000-7000 c.f.u./plate. To each plate was added 3 ml of medium. The bacteria were scraped off, 1 ml glycerol was added and stored at -80°C as pools. The remaining 2 ml were used for DNA isolation. For further details on this method, reference is made to WO 94/14952.

Construction of Yeast Libraries

To ensure that all the bacterial clones were tested in yeast, a number of yeast transformants 5 times larger than the number of bacterial clones in the original pools was set as the limit.

One μl aliquot of purified plasmid DNA (100 ng/μl) from individual pools were electrophorated (200 Ω, 1.5 kV, 25 μF) into 40 μl competent *Saccharomyces cerevisiae* JG169 cells (OD_{600} = 1.5 in 500 ml YPD, washed twice in cold DIW, once in cold 1 M sorbitol, resuspended in 0.5 ml 1 M sorbitol (*Becker D M, Guarante L,* Methods Enzymol. 1991 194 182-187). After addition of 1 ml 1M cold sorbitol, 80 μl

aliquot were plated on SC + glucose - uracil to give 250-400 c.f.u./plate and incubated at 30°C for 3-5 days.

Identification of Positive Colonies

After 3-5 days of growth, the agar plates were replica plated onto SC-Uracil plates containing 0.2% Azurin-cross-linked birch xylan (AZCL™ birch xylan, Megazyme™, Australia), and 2% galactose, followed by incubation for 2-4 days at 30°C for detection of xylanolytic activity. After incubation xylanolytic enzyme-positive colonies were identified as colonies with a blue halo around.

Cells from enzyme-positive colonies were spread for single colony isolation on agar, and an enzyme-producing single colony was selected for each of the xylanolytic enzyme-producing colonies identified.

Characterization of Positive Clones

The positive clones were obtained as single colonies. Plasmid DNA was isolated from a cell culture prepared from the two positive yeast colonies. Plasmid DNA was introduced (transformed) into *E. coli*, isolated and characterized individually by sequencing the 5'-end of each cDNA clone using the chain-termination method (*Sanger et al.*, Proc. Natl. Acad. Sci. U. S. A. 1977 74 5463-5467), and the Sequenase™ System (United States Biochemical).

Isolation of a cDNA gene for Expression in *Aspergillus*

One or more xylanolytic enzyme-producing yeast colonies were inoculated into 20 ml YNB-1 broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

DNA, isolated according to WO 94/14952, was dissolved in 50 µl water to a final concentration of approximately 100 µl/ml. Aliquot of the DNA were transformed with *E. coli* as described in WO 94/14952. Plasmid DNA was isolated from *E. coli* using standard procedures, and analyzed by restriction enzyme analysis. The cDNA insert was excised using appropriate restriction enzymes and ligated into an *Aspergillus* expression vector.

Transformation of *Aspergillus oryzae* or *Aspergillus niger*

General Procedure

100 ml of YPD (*Sherman et al.*, Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) is inoculated with spores of *A. oryzae* or *A. niger* and incubated with shaking at 37°C for about 2 days. The mycelium is harvested by filtration and washed with 200 ml of 0.6 M $MgSO_4$. The mycelium is suspended in 15 ml of 1.2 M $MgSO_4$ and 10 mM NaH_2PO_4, pH 5.8. The suspension is cooled on ice and 1 ml of buffer containing 120 mg of Novozym™ 234, batch 1687 is added. After 5 minutes 1 ml of 12 mg/ml BSA (Sigma, type H25) is added and incubation with gentle agitation continued for 1.5-2.5 hours at 37°C until a large number of protoplasts is visible in a sample inspected under the microscope.

The suspension is filtered through Miracloth, the filtrate transferred to a sterile tube and overlayered with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH 7.0. Centrifugation is performed for 15 minutes at 100 g and the protoplasts are collected from the top of the $MgSO_4$ cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$) are added to the protoplast suspension and the mixture is centrifuged for 5 minutes at 1000 g. The protoplast pellet is resuspended in 3 ml of STC and repelleted. This is repeated. Finally the protoplasts are resuspended in 0.2-1 ml of STC.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC. Protoplasts are mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576). 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5, is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on the appropriate plates. Protoplasts are spread on minimal plates (*Cove*, Biochem. Biophys. Acta 1966 113 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is

repeated and spores of a single colony after the second reisolation is stored as a defined transformant.

Test of *Aspergillus oryzae* Transformants

Each of the transformants were inoculated in 10 ml of YPM (cf. below) and propagated. After 2-5 days of incubation at 30°C, the supernatant was removed. The xylanolytic activity was identified by applying 10 µl supernatant to 4 mm diameter holes punched out in agar plates containing 0.2% AZCL™ birch xylan (Megazyme™, Australia). Xylanolytic activity is then identified as a blue halo.

Media

YPM media: 10 g yeast extract, 20 g peptone, H_2O to 810 ml. 90 ml 20% maltodextrin, autoclaved and sterile filtered, is added.

YPD media: 10 g yeast extract, 20 g peptone, H_2O to 810 ml. 90 ml 20% glucose, autoclaved and sterile filtered, is added.

10 x Basal salt media: 66.8 g yeast nitrogen base, 100 g succinic acid, 60 g NaOH, H_2O ad 1000 ml, sterile filtered.

SC-URA: 90 ml 10 x Basal salt, 22.5 ml 20% casamino acids, 9 ml 1% tryptophan, H_2O ad 806 ml, autoclaved, 3.6 ml 5% threonine and 90 ml 20% glucose or 20% galactose added.

SC-H broth: 7.5 g/l yeast nitrogen base without amino acids, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids without vitamins, 0.1 g/l tryptophan. Autoclaved for 20 min. at 121°C. After autoclaving, 10 ml of a 30% galactose solution, 5 ml of a 30% glucose solution and 0.4 ml of a 5% threonine solution were added per 100 ml medium.

SC-H agar: 7.5 g/l yeast nitrogen base without amino acids, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids without vitamins, 0.1 g/l tryptophan, and 20 g/l agar (Bacto™). Autoclaved for 20 min. at 121°C. After autoclaving, 55 ml of a 22% galactose solution and 1.8 ml of a 5% threonine solution were added per 450 ml agar.

YNB-1 agar: 3.3 g/l KH_2PO_4, 16.7 g/l agar, pH adjusted to 7. Autoclaved for 20 min. at 121°C. After autoclaving, 25 ml of a 13.6% yeast nitrogen base without

amino acids, 25 ml of a 40% glucose solution, 1.5 ml of a 1% L-leucine solution and 1.5 ml of a 1% histidine solution were added per 450 ml agar.

YNB-1 broth: Composition as YNB-1 agar, but without the agar.

Isolation of the Gene

A library from *Thermomyces lanuginosus* consisting of approx. 1.5 x 10^6 individual clones in 150 pools was constructed. DNA was isolated from 20 individual clones from the library and subjected to analysis for cDNA insertion. The insertion frequency was found to be >90% and the average insert size was approximately 1400 bp.

DNA from some of the pools was transformed into yeast, and 50-100 plates containing 200-500 yeast colonies were obtained from each pool. After 3-5 days of growth, the agar plates were replica plated onto several sets of agar plates. One set of plates containing 0.1% AZCL™ xylan (Megazyme™, Australia) was then incubated for 3-5 days at 30°C to detect for xylanase activity. Positive colonies were identified as colonies surrounded by a blue halo. Alternatively, one set of plates was then incubated for 3-5 days at 30°C before over-layering with a xylan overlayer gel containing 0.1% AZCL™ xylan and 1% agarose in a buffer with an appropriate pH. After incubation for 1-2 days at 30°C, positive colonies were identified as colonies surrounded by a blue halo.

Cells from enzyme-positive colonies were spread for single colony isolation on agar, and an enzyme-producing single colony was selected for each of the xylanase-producing colonies identified.

Characterization of Positive Clones

The positive clones were obtained as single colonies. cDNA inserts were amplified directly from the yeast colony using biotinylated polylinker primers, purified by magnetic beads (Dynabead™ M-280, Dynal) system and characterized individually by sequencing the 5'-end of each cDNA clone using the chain-termination method (*Sanger F, Nicklen S & Coulson A R*; Proc. Natl. Acad. Sci. U. S. A. 1977 74 5463-5467) and the Sequenase™ System (United States Biochemical).

The DNA sequence is shown as SEQ ID NO: 1, which corresponds to the amino acid sequence presented as SEQ ID NO: 2.

Isolation of Yeast DNA

In order to avoid PCR errors in the gene to be cloned, the cDNA was isolated from the yeast plasmids by standard procedures, e.g. as described in Example 1 of WO 93/11249, which publication is hereby included by reference. The yeast DNA was dissolved in 50 µl water to a final concentration of approximately 100 µl/ml.

The DNA was transformed into *Escherichia coli* by standard procedures. Two *E. coli* colonies were isolated from each of the transformations and analyzed with the restriction enzymes HindIII and XbaI which excised the DNA insert. DNA from one of these clones was retransformed into yeast strain JG169.

The DNA sequences of several of the positive clones were partially determined. The DNA sequences of the xylanolytic enzyme according to the invention is shown as SEQ ID NO: 1, which corresponds to the amino acid sequence presented as SEQ ID NO: 2.

Expression in *Aspergillus oryzae*

In order to express the gene in *Aspergillus*, cDNA is isolated from one of the above clones by digestion with HindIII/XbaI or other appropriate restriction enzymes, size fractionation on a gel and purification and subsequently ligated to pHD414, resulting in plasmids pA2XITI. After amplification in *E. coli*, the plasmid is transformed into a strain of *Aspergillus oryzae* according to the general procedure described above.

Test of *Aspergillus oryzae* Transformants

Each of the transformants were inoculated in 10 ml YPM medium. After 3-5 days of incubation at 30°C and 250 rpm, the supernatant was removed. The xylanolytic activity was determined by applying 10 µl supernatant into 4 mm (diameter) holes punched in an agar plate containing 0.2% AZCL™ xylan (Megazyme™, Australia) in a buffer with an appropriate pH, and incubated overnight at

40°C. The xylanase activity was identified as described above. Some of the transformants had halos which were significantly larger than the *Aspergillus oryzae* background. This demonstrates efficient expression of xylanase in *Aspergillus oryzae*. The 8 transformants with the highest xylanase activity were selected and inoculated and maintained on YPG-agar.

Each of the 8 selected transformants were inoculated from YPG-agar slants on 500 ml shake flask with FG-4 and MDU-2 media. After 3-5 days of fermentation with sufficient agitation to ensure good aeration, the culture broths were centrifuged for 10 minutes at 2000 g and the supernatants were analyzed.

A volume of 15 µl of each supernatant was applied to 4 mm diameter holes punched out in a 0.1% AZCL™ xylan overlayer gel (25 ml in a 13 cm diameter petri dish). The xylanase activity was identified by the formation of a blue halo on incubation.

Subsequently, the xylanase was fermented in a medium comprising maltodextrin as a carbon source, urea as a nitrogen source and yeast extract. The fermentation was performed by innoculating a shake flask culture of the *Aspergillus oryzae* host cells into a medium comprising 3.5% of the carbon source and 0.5% of the nitrogen source. After 24 hours of cultivation at pH 5.0 and 34°C the continuous supply of additional carbon and nitrogen sources were initiated. The carbon source was kept as the limiting factor and it was secured that oxygen was present in excess. The cultivation was continued for 4 days, after which the enzymes could be recovered by centrifugation, ultrafiltration, clear filtration and germ filtration.

Purification

The culture supernatant from fermentation of *Aspergillus oryzae*, described above, expressing the recombinant enzyme is centrifugated and filtered through a 0.2µm filter to remove the mycelia.

100 ml of the filtered supernatant is ultra-filtrated in a Filtron™ ultracette or Amicon™ ultrafiltration device with a 3 kDa membrane to achieve 10 fold concentration. This concentrate is diluted 100 times in 20 mM TRIS, pH 8.0, in two successive rounds of ultrafiltration in the same device. This ultrafiltration sample is

loaded at 2 ml/min on a Pharmacia XK 26/20 Fast Flow Q Sepharose™ anion exchanger, equilibrated in 20 mM TRIS, pH 8.0.

After the sample has been applied, the column is washed with two column volumes 25 mM TRIS, pH 8.0, and bound proteins are eluted with a linear increasing NaCl gradient from 0 to 0.5 M NaCl in 25 mM TRIS, pH 8.0. Fractions are collected and the xylanase activity in the fractions measured as described above.

Xylanase containing fractions are pooled and UF concentrated into 10 mM sodium citrate, pH 4.0. This material is loaded on a XK 16/20 Fast Flow S Sepharose™ column at 1.5 ml/min. The enzyme is eluted with a linear gradient from 0 to 0.4 M NaCl and xylanase containing fractions pooled, concentrated and used for characterization and further experimentation as described below.

EXAMPLE 2

Enzyme Characterization

The xylanolytic enzyme obtained according to Example 1 was subjected to the following enzyme characterization.

SDS-PAGE Electrophoresis

SDS-PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis) was performed in a Mini-Leak 4 electrophoresis unit (Kem-En-Tec, Copenhagen) as a modified version of the Laemmli procedure (*Laemmli U K*; Nature 1970 227, 680-685; *Christgau et al.*, 1991, J. Biol. Chem. 1991 266 p. 21157-212664].

A molecular weight (MW) of approximately 26 kDa was determined.

Isoelectric Focusing

Isoelectric focusing was carried out on Ampholine™ PAG plates, pH 3.5-9.5 (Pharmacia, Sweden) on a Multiphor™ electrophoresis unit according to the manufactures instructions. After electrophoresis, the gel was commassie stained according to standard protocols known in the art.

An isoelectric point (pI) of approximately 4.5 was determined.

pH and Temperature Optima

Enzymatic activities are measured by the release of blue color from AZCL™ birch xylan (Megazyme, Australia).

0.5 ml 0.4% AZCL™ substrate suspension is mixed with 0.5 ml 0.1M citrate/phosphate buffer of optimal pH, and 10 µl of a suitably diluted enzyme solution is added. Incubations are carried out in Eppendorph Thermomixers for 15 minutes at 30°C, followed by heat inactivation for 20 minutes at 95°C. Enzyme incubations are carried out in triplicate. A blank is produced in which enzyme is added but inactivated immediately. After centrifugation, the absorbance of the supernatant is measured in microtiter plates at 620 nm and the blank is subtracted.

0.1 M citrate/phosphate buffers of varying pH were used for determination of pH optimum. A 0.1M citrate/phosphate buffer, pH 5.5, for incubation at different temperatures for 15 minutes was used in order to determine the temperature optimum.

By determination of the relative xylanolytic activity (%) at 30°C in the range of from pH 2.5 to 9, it was found that the enzyme has a pH optimum in the range 4.5-7.5, more specifically the range 5.0-6.5, around pH 6.

By determination of the relative xylanolytic activity (%) at pH 5.5 in the range 30 to 80°C, it was found that the enzyme has a temperature optimum in the range 50-70°C, around 60°C.

EXAMPLE 3

Baking Trials

This example demonstrates the bread-improving properties of an additive of the invention. The *Thermomyces lanuginosus* xylanase was obtained according to Example 1, and dosed in FXU activity units, as defined above.

For making the dough, the following recipe was used (ingredients in % w/w of flour):

Water	60.0
Flour (Intermill Type 550)	100
Yeast	4
Salt	1.5
Sugar	1.5
Ascorbic acid (20 ppm)	0.002

The ingredient were scaled and transferred to the mixer.

After 3 and 6 minutes of mixing, respectively (slow and fast, 750 and 1500 RPM), and 15 minutes of fermentation at 28°C and 60% relative humidity, the dough was scaled into portions of 350 g.

After 5 minutes of sheeting/moulding and relaxing on the table for 5 minutes, followed by a second 5 minutes steeting/shoulding step, the dough was transferred to the pan and fermented for 55 minutes (70 minutes for overfermented bread) at 32°C and 80% relative humidity.

Breads were baked for 35 minutes at 235°C (30 minutes at 230°C for overfermented bread).

The results are presented in Tables 1 and 2, below.

Table 1

Evaluation of the Dough and Normal Fermented Bread

Enzyme dosage FXU/kg flour	150	250	350	450	Control
Average weight g	300	302	299	299	304
Average volume ml	1508	1558	1610	1615	1475
Specific volume ml/g	5.03	5.16	5.38	5.41	4.85
Specific volume % relative	104	106	111	112	100
Color	3.5	4	4	4	3
Crust crispy	3	4	4.5	4.5	3
Crumb structure	3	3.5	3.5	3	3
Evaluation of Dough					
Stickiness	4	3.5	3.5	3	4
Firmness	4	3.5	3.5	3	4
Extensibility	3	3.5	4	4	3.5
Elasticity	3	3.5	3.5	4	3

Bread evaluation scale:

Color: 1 very light, 2 light, 3 normal, 4 good, 5 very good, 6 too dark.

Crust crispy: 1 too rubbery, 2 rubbery, 3 normal, 4 good/crispy, 5 very good, 6 too crispy.

Crumb structure: 1 very poor, 2 poor/non uniform, 3 normal, 4 good/uniform, 5 very good.

Dough evaluation scale:

Stickiness: 1 almost liquid, 2 too sticky, 3 sticky, 4 good, 5 dry, 6 too dry.

Firmness: 1 very soft, 2 too soft, 3 normal, 4 good, 5 long, 6 too long.

Extensibility: 1 too strong, 2 strong, 3 normal, 4 good, 5 weak, 6 too weak.

Table 2

Evaluation of Overfermented Bread

Enzyme dosage FXU/kg flour	150	250	350	450	Control
Average weight g	299	300	298	303	302
Average volume ml	1650	1555	1690	1755	1410
Specific volume ml/mg	5.53	5.19	5.67	5.80	4.87
Specific volume % relative	118	111	121	124	100

The results of these baking trials clearly demonstrate the excellent bread-improving properties of an additive of the invention.

When added to the dough, the xylanolytic enzyme of the invention improves bread volume and evaluation score on color and crust crispiness. Furthermore, the xylanolytic enzyme has a positive effect on dough stability, as seen from the specific volume index on breads overfermented and shocked, as well as dough extensibility and elasticity. It is also noticeable that the optimal volumeimprovement is obtained before dough stickiness has become unacceptable.

SEQUENCE LISTING

INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 983 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Thermomyces lanuginosus

(B) STRAIN: DSM 4109

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:31..705

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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TCGGCCCGAC GTCTTGCAAT CCTTGCAGTG ATG GTC GGC TTT ACC CCC GTT GCC      54
                                 Met Val Gly Phe Thr Pro Val Ala
                                   1               5

CTT GCG GCC TTA GCC GCG ACT GGG GCC CTG GCC TTC CCG GCA GGG AAT      102
Leu Ala Ala Leu Ala Ala Thr Gly Ala Leu Ala Phe Pro Ala Gly Asn
  10                  15                  20

GCC ACG GAG CTC GAA AAG CGA CAG ACA ACC CCC AAC TCG GAG GGC TGG      150
Ala Thr Glu Leu Glu Lys Arg Gln Thr Thr Pro Asn Ser Glu Gly Trp
 25                  30                  35                  40

CAC GAT GGT TAT TAC TAT TCC TGG TGG AGT GAC GGT GGA GCG CAG GCC      198
His Asp Gly Tyr Tyr Tyr Ser Trp Trp Ser Asp Gly Gly Ala Gln Ala
                 45                  50                  55

ACG TAC ACC AAC CTG GAA GGC GGC ACC TAC GAG ATC AGC TGG GGA GAT      246
Thr Tyr Thr Asn Leu Glu Gly Gly Thr Tyr Glu Ile Ser Trp Gly Asp
         60                  65                  70

GGC GGT AAC CTC GTC GGT GGA AAG GGC TGG AAC CCC GGC CTG AAC GCA      294
Gly Gly Asn Leu Val Gly Gly Lys Gly Trp Asn Pro Gly Leu Asn Ala
       75                  80                  85

AGA GCC ATC CAC TTT GAG GGT GTT TAC CAG CCA AAC GGC AAC AGC TAC      342
Arg Ala Ile His Phe Glu Gly Val Tyr Gln Pro Asn Gly Asn Ser Tyr
  90                  95                  100
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CTT GCG GTC TAC GGT TGG ACC CGC AAC CCG CTG GTC GAG TAT TAC ATC      390
Leu Ala Val Tyr Gly Trp Thr Arg Asn Pro Leu Val Glu Tyr Tyr Ile
105                 110                 115                 120

GTC GAG AAC TTT GGC ACC TAT GAT CCT TCC TCC GGT GCT ACC GAT CTA      438
Val Glu Asn Phe Gly Thr Tyr Asp Pro Ser Ser Gly Ala Thr Asp Leu
                125                 130                 135

GGA ACT GTC GAG TGC GAC GGT AGC ATC TAT CGA CTC GGC AAG ACC ACT      486
Gly Thr Val Glu Cys Asp Gly Ser Ile Tyr Arg Leu Gly Lys Thr Thr
            140                 145                 150

CGC GTC AAC GCA CCT AGC ATC GAC GGC ACC CAA ACC TTC GAC CAA TAC      534
Arg Val Asn Ala Pro Ser Ile Asp Gly Thr Gln Thr Phe Asp Gln Tyr
        155                 160                 165

TGG TCG GTC CGC CAG GAC AAG CGC ACC AGC GGT ACC GTC CAG ACG GGC      582
Trp Ser Val Arg Gln Asp Lys Arg Thr Ser Gly Thr Val Gln Thr Gly
    170                 175                 180

TGC CAC TTC GAC GCC TGG GCT CGC GCT GGT TTG AAT GTC AAC GGT GAC      630
Cys His Phe Asp Ala Trp Ala Arg Ala Gly Leu Asn Val Asn Gly Asp
185                 190                 195                 200

CAC TAC TAC CAG ATC GTT GCA ACG GAG GGC TAC TTC AGC AGC GGC TAT      678
His Tyr Tyr Gln Ile Val Ala Thr Glu Gly Tyr Phe Ser Ser Gly Tyr
                205                 210                 215

GCT CGC ATC ACC GTT GCT GAC GTG GGC TAAGACGTAA CCTGGTGGTG            725
Ala Arg Ile Thr Val Ala Asp Val Gly
            220                 225

ATCTCGCGAG GCAACAGCCA AGAATGTCGT CAGATGTGCC GGTTGAAGGT ATTCAATCAG    785

CATATCTGTC TGCCCTTGCG AGTGATACTT TGGAGGACTG TGGAGAACTT TGTGCGAGCC    845

TGGCCAGGAT CAGTAGTTGC TTTGCGGTGT TTTGCTCCCT ATTCTCGTGA AAAAATTGTT    905

ATTGCTTCGT TGTCTAGTGT ACATAGCCGA GCAATTGAGG CCTCACGCTT GGGAAAAAAA    965

AAAAAAAAAA AAAAAAAA                                                  983
```

INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 225 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Val Gly Phe Thr Pro Val Ala Leu Ala Ala Leu Ala Ala Thr Gly
1 5 10 15

Ala Leu Ala Phe Pro Ala Gly Asn Ala Thr Glu Leu Glu Lys Arg Gln
20 25 30

Thr Thr Pro Asn Ser Glu Gly Trp His Asp Gly Tyr Tyr Tyr Ser Trp
35 40 45

Trp Ser Asp Gly Gly Ala Gln Ala Thr Tyr Thr Asn Leu Glu Gly Gly
50 55 60

Thr Tyr Glu Ile Ser Trp Gly Asp Gly Gly Asn Leu Val Gly Gly Lys
65 70 75 80

Gly Trp Asn Pro Gly Leu Asn Ala Arg Ala Ile His Phe Glu Gly Val
85 90 95

Tyr Gln Pro Asn Gly Asn Ser Tyr Leu Ala Val Tyr Gly Trp Thr Arg
100 105 110

Asn Pro Leu Val Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr Asp
115 120 125

Pro Ser Ser Gly Ala Thr Asp Leu Gly Thr Val Glu Cys Asp Gly Ser
130 135 140

Ile Tyr Arg Leu Gly Lys Thr Thr Arg Val Asn Ala Pro Ser Ile Asp
145 150 155 160

Gly Thr Gln Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Asp Lys Arg
165 170 175

Thr Ser Gly Thr Val Gln Thr Gly Cys His Phe Asp Ala Trp Ala Arg
180 185 190

Ala Gly Leu Asn Val Asn Gly Asp His Tyr Tyr Gln Ile Val Ala Thr
195 200 205

Glu Gly Tyr Phe Ser Ser Gly Tyr Ala Arg Ile Thr Val Ala Asp Val
210 215 220

Gly
225

CLAIMS

1. A bread-improving additive, comprising a xylanolytic enzyme derived from a strain of *Thermomyces*.

2. The bread-improving additive according to claim 1, in which the xylanolytic enzyme is derived from a strain of *Thermomyces lanuginosus*.

3. The additive according to either of claims 1-2, in which the xylanolytic enzyme is derived from the strain *Thermomyces lanuginosus* DSM 4109, or a mutant or a variant thereof.

4. The additive according to any of claims 1-3, in which the xylanolytic enzyme has the amino acid sequence presented as SEQ ID NO: 2, or any partial sequence hereof.

5. The additive according to any of claims 1-3, in which the xylanolytic enzyme has an amino acid sequence homologue to the sequence presented as SEQ ID NO: 2.

6. The additive according to any of claims 1-5, in which the xylanolytic enzyme is provided in the form of a monocomponent xylanase preparation.

7. The additive according to claim 6, in which the monocomponent xylanase is

(a) encoded by the DNA sequence presented as SEQ ID NO: 1, or by the DNA sequence obtainable from the plasmid in the strain *Saccharomyces cerevisiae* DSM 10133; or

(b) encoded by a DNA sequence analogue to the xylanase encoding part of the DNA sequence presented as SEQ ID NO: 1, or to the DNA sequence obtainable from the plasmid in the strain *Saccharomyces cerevisiae* DSM 10133, which analog DNA sequence either

(i) is homologous to the xylanase encoding part of the DNA sequence presented as SEQ ID NO: 1, or to the DNA sequence obtainable from the plasmid in the strain *Saccharomyces cerevisiae* DSM 10133; or

(ii) hybridizes with the same oligonucleotide probe as the xylanase encoding part of the DNA sequence presented as SEQ ID NO: 1, or with the DNA sequence obtainable from the plasmid in the strain *Saccharomyces cerevisiae* DSM 10133; or

(iii) encodes a polypeptide which is at least 70% homologous to the polypeptide encoded by the DNA sequence presented as SEQ ID NO: 1, or to the DNA sequence obtainable from the plasmid in the strain *Saccharomyces cerevisiae* DSM 10133; or

(iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified xylanase derived from the strain *Thermomyces lanuginosus*, DSM 4109, or encoded by the DNA sequence presented as SEQ ID NO: 1, or the DNA sequence obtainable from the plasmid in the strain *Saccharomyces cerevisiae* DSM 10133.

8. The additive according to any of claims 1-7, in which the xylanolytic enzyme is added in an amount corresponding to of from about 5 to about 5000 FXU/kg of flour, preferably of from about 20 to about 2000 FXU/kg of flour.

9. The additive according to any of claims 1-8, which comprises one or more additional enzymes selected from the group consisting of an amylase, a maltogenase, a lipase, a cellulase, a hemicellulase, a pentosanase, a glucose oxidase, a laccase, a protease and a peroxidase.

10. The additive according to any of claims 1-8, which comprises one or more additional enzymes selected from the group consisting of a lipase, an amylase and an oxidase.

11. The additive according to any of claims 1-8, which comprises one or more additional enzymes selected from the group consisting of an α-amylase and an amyloglucosidase.

12. The additive according to any of claims 1-8, which comprises a lipase.

13. The additive according to any of claims 1-8, which comprises an amylase.

14. The additive according to claim 13, in which the amylase is an α-amylase.

15. The additive according to claim 14, in which the α-amylase is of fungal origin.

16. The additive according to claim 15, in which the α-amylase is derived from a strain of *Aspergillus*.

17. The additive according to claim 16, in which the α-amylase is derived from a strain of *Aspergillus oryzae*.

18. A method of preparing a baked product, which method comprises adding to the flour or to the dough the bread-improving additive according to any of claims 1-17.

19. A method of improving the baking properties of flour and/or dough, which method comprises adding to the flour and/or to the dough the bread-improving additive according to any of claims 1-17.

20. The method according to claim 18, which method comprices adding the bread-improving additive according to any of claims 1-18 to any ingredient of the dough and/or to any mixture of the dough ingredients.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00171

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/42, A21D 8/04
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, A21D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, FSTA, WPI, WPIL, CA, US PATENTS FULLTEXT, SCISEARCH, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0507723 A1 (NOVO NORDISK A/S), 7 October 1992 (07.10.92), page 3, line 6 - line 8; page 3, line 25 - line 31; page 3, line 48 - line 49, the claims --	1-20
X	WO 9421785 A1 (NOVO NORDISK A/S), 29 Sept 1994 (29.09.94), page 8, line 12; page 13, line 11 - line 26, claim 15 --	1-20
X	EP 0396162 A1 (UNILEVER NV), 7 November 1990 (07.11.90), page 3, line 3 - line 4, the claims --	1-20

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 July 1996	18 -07- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00171

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, File 5, Biosis, Dialog accession no. 10989020, Alam M. Gomes et al: "Production and characterization of thermostable xylanases by Thermomyces lanuginosus and Thermoascus aurantiacus grown on lignocelluloses", Enzyme and Microbial Technology 16 (4).1994. 298-302 -- --------	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00171

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1 and 7
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

See extra sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

The wording "...mutant or variant thereof" of claim 3 is not considered to be clear and concise since the mutant or variant is not restricted to possess the intended, special features of the parent xylanolytic enzyme.

The wording "homologous" of claim 7 (i) is not considered to be clear and concise since it has not been specified to what extent the sequence is homologous to the xylanase encoding part of the DNA sequence presented as SEQ ID NO:1. It should be clear from the claim that the part(s) of SEQ ID NO:1 that encodes the alleged inventive features of the xylanase is present in the analogue which is defined "homologous".

Thw wording "immunologically reactive with an antibody raised against" of claim 7 (iv) does not define a property that is relevant in the context of the invention, as there is no direct link between the xylanase activity and the immunological features (except for some unknown epitopes of the active site).

Consequently, claims 3 and 7 are not considered to fulfil the requirements of PCT Article 6 regarding clarity and conciseness.

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/04/96

International application No.
PCT/DK 96/00171

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0507723	07/10/92	CA-A- 2106484	03/10/92
		EP-A- 0579672	26/01/94
		JP-T- 6506348	21/07/94
		WO-A,A- 9217573	15/10/92
WO-A1- 9421785	29/09/94	NONE	
EP-A1- 0396162	07/11/90	SE-T3- 0396162	
		AU-B,B- 617934	05/12/91
		AU-A- 5206190	27/09/90
		CA-A- 2012723	23/09/90
		DE-U- 6900072	18/02/93
		DE-T- 69000722	01/02/96
		ES-T,T- 2054212	01/08/94
		JP-C- 1762949	28/05/93
		JP-A- 3035749	15/02/91
		JP-B- 4057302	11/09/92
		US-A- 5108765	28/04/92